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# Increased Expression of Lysine-Specific Demethylase 5B (KDM5B) Promotes Tumor Cell Growth in Hep3B Cells and is an Independent Prognostic Factor in Patients with Hepatocellular Carcinoma

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Data Collection B  
Statistical Analysis C  
Data Interpretation D  
Manuscript Preparation E  
Literature Search F  
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**Background:** Lysine-specific demethylase 5B (KDM5B) is overexpressed in several types of cancer. However, the clinical significance of KDM5B expression in hepatocellular carcinoma (HCC) remains unclear. The aims of the present study were to examine the functional effects of KDM5B in the Hep3B cell line, the expression levels of KDM5B in human HCC tissues, and the association between KDM5B expression and clinical outcome in patients with HCC.


**Material/Methods:** Immunohistochemistry (IHC) and quantitative real-time polymerase chain reaction (qRT-PCR) were used to examine the expression levels of KDM5B in HCC tissues and adjacent normal liver tissues. In the HCC cell line, Hep3B, the effects of KDM5B on cell proliferation and migration, and KDM5B small interfering RNA (siRNA) were used to study KDM5B knockdown. Univariate and multivariate analysis assessed the prognostic role of KDM5B in HCC patients. Kaplan-Meier analysis and the log-rank test evaluated clinical outcomes.

**Results:** In the HCC cell line, Hep3B, KDM5B expression promoted tumor cell proliferation and colony formation. Increased expression of KDM5B in HCC tissues, compared with adjacent normal liver tissues, and was associated with larger tumor size, advanced TNM stage, and reduced overall survival in patients with HCC. Multivariate analysis identified KDM5B expression as an independent prognostic factor.

**Conclusions:** Increased expression of KDM5B was significantly correlated with poorer prognosis in patients with patients with HCC, indicating the possible potential of KDM5B as a novel clinical biomarker and therapeutic target.

**MeSH Keywords:** **Carcinoma, Hepatocellular • Cell Proliferation • Prognosis**

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## Background

Worldwide, hepatocellular carcinoma (HCC) is the main type of primary liver cancer and is the third most common cause of cancer-related death [1]. Current treatment options for patients with HCC include surgical resection, chemotherapy, radiotherapy and immunotherapy [2]. Despite the recent progress in early diagnosis and treatment, patients with HCC still have an aggressive clinical course and poor prognosis, especially from advanced-stage disease, with less than 6% of patients with HCC surviving more than 5 years [3]. Studies on the molecular signaling pathways involved in the pathogenesis of HCC have been undertaken, but the mechanisms underlying the development of HCC remain poorly understood [4]. Therefore, there is still a need to investigate the mechanisms involved in the pathogenesis of HCC and to identify potential prognostic biomarkers, more effective treatment strategies, as well as developing new therapeutic targets.

Epigenetic regulation plays multiple roles in a wide variety of physiological processes [5,6]. The homeostasis of epigenetic states is essential for the maintenance of chromatin structure and cell-specific functions [7]. Recent studies have shown that epigenetic alterations, including modifications in histones, are significantly associated with the development of several human malignancies, including osteosarcoma, glioma, and gastric adenocarcinoma [8,9]. As one of the most important types of histone modification, histone methylation contributes significantly to the regulation of chromatin structure and gene expression [10]. For example, lysine methylation on H3K4 is associated with transcriptional activation, whereas lysine methylation on H3K9 is associated with transcriptional repression [11]. It has now become acknowledged that alterations in histone methylation play important roles in the initiation and progression of cancer [12].

Lysine-specific demethylase 5 (KDM5), also known as JARID1, consists of four family members, KDM5A, KDM5B, KDM5C, and KDM5D [13]. The KDM5 family contains five conserved domains: the catalytic JmjC domain, N-terminal JmjN domain, ARID domain, PHD finger domain, and the C5CH2 domain [14]. It has been reported that tumor cells can utilize KDM5A to acquire chemotolerance to cytotoxic agents, such as cisplatin, which suggests a potential role of KDM5A in cancer therapeutics [15]. Although KDM5B is found to be primarily expressed in the normal testis, expression levels of KDM5B have been shown to be significantly upregulated in several human malignancies, including gastric carcinoma, glioma, and malignant osteosarcoma [16]. Also, KDM5B has been shown to have a role in tumor initiation and progression [17].

Therefore the aims of the present study were to examine the functional effects of KDM5B in the Hep3B human HCC cell line,

the expression levels of KDM5B in human HCC tissues compared with adjacent normal liver tissues. The prognostic role of KDM5B expression was evaluated with univariate and multivariate analysis. Kaplan-Meier analysis and the log-rank test evaluated clinical outcomes associated with expression of KDM5B on tumor tissues from patients with HCC.

## Material and Methods

### Reagents

The anti-KDM5B (ab181089) and anti-beta actin (ab8227) antibodies were purchased from Abcam. The KDM5B small interfering RNA (siRNA) was purchased from ABM (Richmond, BC, Canada). The Lipofectamine 3000 Reagent was purchased from Invitrogen (Carlsbad, CA, USA). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.

### Patients and samples

This study was approved by the Ethics Committee of the Third Xiangya Hospital of Central South University. Written informed consent was obtained from all study participants. There were 152 pairs of formalin-fixed paraffin-embedded hepatocellular carcinoma (HCC) tissues and adjacent normal tissues selected from patients who underwent surgical resection in The Third Xiangya Hospital. Also, another 21 pairs of fresh HCC tissues together with adjacent normal liver tissues were obtained from the Department of Surgery, which were fresh-frozen with liquid nitrogen for mRNA analysis. All enrolled patients had no history of previous radiotherapy or chemotherapy and underwent followed-up until death or the end of the study, which ranged from between 3–59 months. All the specimens used in the present study underwent histopathology examination.

### Immunohistochemistry (IHC) staining

Immunohistochemistry (IHC) staining for KDM5B was performed using a standard protocol, as previously described [18]. Briefly, 8 µm serial sections were cut onto glass slides, they were dried and then deparaffinized with xylene and rehydrated in alcohols. Microwave antigen retrieval was performed in citrate buffer at pH 6.0. Then, the sections were incubated overnight with the primary rabbit anti-human KDM5B antibody (1: 200 dilution) (Cat. No. ab181089) (Abcam). On the next day, the sections were washed and the localization of the primary antibody was visualized by using horseradish peroxidase (HRP)-conjugated IgG and 3,3'-diaminobenzidine (DAB) (brown) substrate. The negative control replaced the primary antibody with phosphate-buffered saline (PBS).

### Evaluation of IHC staining

The stained slides were examined by light microscopy and scored by two independent investigators at a magnification of  $\times 400$ . Six microscopic fields of each section were randomly selected. Staining intensity was divided into four grades, as follows: 1 (weak); 2 (moderate); 3 (strong). The percentage of positively-stained cells was scored as follows: 1 ( $<25\%$ ); 2 (25–50%); 3 (51–75%); 4 ( $>75\%$ ). The sum of intensity score and staining percentage score was defined as the final IHC score. To better evaluate the clinical significance of KDM5B in HCC, patients were divided into a high KDM5B expression group (IHC score  $\geq 5$ ) (N=80) and a low KDM5B expression group (IHC score  $<5$ ) (N=70).

### Western blot

The transfected cells were washed with PBS and lysed in cold lysis buffer. The cell lysates were centrifuged at  $14,000\times g$  at  $4^{\circ}\text{C}$  for 30 min. The supernatant was collected and denatured in the loading buffer. After protein quantification, equal amounts of total protein were separated using a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were immunoblotted with specific primary antibodies to KDM5B and  $\beta$ -actin at  $4^{\circ}\text{C}$  overnight and subsequently incubated with the secondary antibodies for a further 2 hours at room temperature. The protein bands were visualized with the enhanced chemiluminescence (ECL) substrate, using  $\beta$ -actin as the loading control.

### RNA preparation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total mRNA was isolated from fresh-frozen specimens using Trizol reagent (Invitrogen, Carlsbad, USA), following a standard protocol [19]. RNA was reversely transcribed into cDNA by using the Primer-Script RT Enzyme Mix (Invitrogen, Carlsbad, CA, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the SYBR Premix Ex Tag (Takara, Japan) according to the manufacturer's instructions.

GAPDH was used as the normal control, and the following primers were used:

*KDM5B*-Forward: 5'-CAGCCCCGACGAGCAAAA-3'

*KDM5B*-Reverse: 5'-CGTTGTCTCTCGGGTTCTATT-3'

*GAPDH*-Forward: 5'-TGCACCACCACTGCTTAGC-3'

*GAPDH*-Reverse: 5'-GGCATGGACTGTGGTCATGAG-3'

### Cell culture and transfection

The hepatocellular carcinoma (HCC) cell line, Hep3B was purchased from the American Type Culture Collection (ATCC)

(Rockville, USA). The overexpression plasmid of KDM5B was synthesized by Genewiz (Suzhou, China) and verified by DNA sequencing. Hep3B cells were transfected with KDM5B plasmid or siRNA by using Lipofectamine 3000 Reagent following the manufacturer's instructions [20].

### Colony formation assay

Transfected cells were seeded into 60 mm wells at a density of 500 cells per well and cultured for 10 days. The colonies were then fixed with methanol for 15 min and stained with crystal violet for 20 min, and colonies containing more than 60 cells were counted. All the experiments were performed in triplicate [21].

### Cell proliferation assay

A cell proliferation assay was performed using the Cell Counting Kit-8 (CCK-8) assay (Dojindo; Japan). Briefly,  $100\ \mu\text{l}$  of cell suspension ( $5\times 10^3$  cells per well) were incubated in 96-well plates and cultured for designated time points, with  $10\ \mu\text{l}$  CCK-8 solution added to each well of the plate and incubated at  $37^{\circ}\text{C}$  for 1 hour. The absorbance was measured at a wavelength of 450 nm using a microplate reader. All the experiments were performed in triplicate.

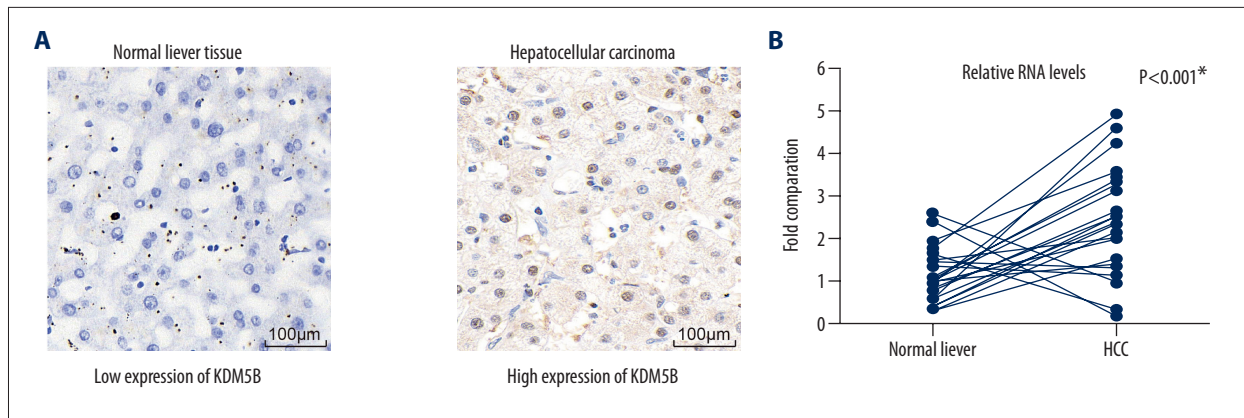
### Statistical analysis

Statistical analysis was performed using SPSS version 19.0 (IBM, New York, NY, USA). The relationship between KDM5B expression and clinical characteristics were assessed using a chi-squared ( $\chi^2$ ) test. The overall survival (OS) curves of patients with HCC were plotted using Kaplan-Meier analysis. Statistical validation of independent prognostic factors was performed with multivariate analysis. Student's t-test was used to analyze the results of cell experiments.  $P<0.05$  was considered to be statistically significant.

## Results

### Expression of KDM5B was increased in human hepatocellular carcinoma (HCC) tissues

To investigate the role of KDM5B in HCC, we first examined its protein level in HCC tissues together with adjacent normal liver tissues by IHC staining. KDM5B was highly expressed in HCC tissues compared with adjacent normal tissues (Figure 1A). The mRNA levels of KDM5B in a further 21 pairs of fresh frozen HCC tissues compared with adjacent normal liver tissues were compared using quantitative real-time polymerase chain reaction (qRT-PCR) assay (Figure 1B). The mRNA level of KDM5B was significantly increased in HCC tissues when compared with adjacent normal liver tissues ( $P<0.001$ ). These results indicated



**Figure 1.** Analysis of KDM5B expression in tissue from patients with hepatocellular carcinoma (HCC). (A) Photomicrographs of the immunohistochemical (IHC) staining for KDM5B in liver tissues. The left panel shows a representative image of low expression of KDM5B in normal adjacent liver tissues; the right panel shows a representative image of high expression of KDM5B in HCC tumor tissues. Magnification  $\times 400$ . (B) mRNA levels of KDM5B were examined in HCC tissues together with adjacent normal liver tissues by quantitative real-time polymerase chain reaction (qRT-PCR). \*  $P < 0.001$  by Student's t-test.

that the KDM5B was highly expressed in HCC tissues and might be involved in the progression of HCC.

#### Increased expression levels of KDM5B indicated poorer clinical outcome in patients with HCC

To investigate the potential role of KDM5B in HCC, using the results from immunohistochemistry (IHC) and tissue staining for KDM5B expression, 72 patients were in the low KDM5B expression group (IHC score  $< 5$ ), and the other 80 HCC patients were in the high KDM5B expression group (IHC score  $\geq 5$ ). Correlations between KDM5B expression and patient clinical outcome were made (Table 1). Increased expression levels of KDM5B expression were significantly associated with increased tumor size ( $P < 0.001$ ) and advanced TNM stage ( $P = 0.012$ ). However, no significant correlations were identified between KDM5B expression and patient age, gender, serum alpha-fetoprotein (AFP), tumor number, histopathological grade, and portal vein invasion (all  $P > 0.05$ ).

#### Increased expression levels of KDM5B were correlated with poor prognosis in patients with HCC

Kaplan-Meier analysis assessed the role of KDM5B expression on overall survival (OS) in patients with HCC and evaluated by the log-rank test (Figure 2). Patients with HCC who expressed higher protein levels of KDM5B had a reduced mean overall survival time ( $30.1 \pm 2.7$  months) compared with patients with HCC who expressed lower protein levels of KDM5B ( $38.5 \pm 2.7$  months) ( $P = 0.018$ ) (Figure 2; Table 2). Some conventional prognostic factors were correlated with the overall survival time in patients with HCC, such as the number of tumor nodules, histopathological grade, portal vein invasion, and TNM stage (all  $P < 0.005$ ) (Figure 2; Table 2).

Multivariate analysis using a Cox hazard regression model was used to test the independent effects of KDM5B on the overall survival of patients with HCC (Table 3). All the factors that showed statistical significance in univariate analysis were enrolled in the Cox regression model, including tumor number, histopathological grade, portal vein invasion, TNM stage, and KDM5B levels. Accordingly, KDM5B expression was identified as an independent prognostic factor (HR=1.686; 95% CI, 1.076–2.642) ( $P = 0.023$ ). Also, the presence of portal vein invasion ( $P = 0.015$ ), and advanced TNM stage ( $P = 0.044$ ) were also found to be independent factors of reduced overall survival in patients with HCC.

#### Increased expression of KDM5B promoted colony formation and cell proliferation of the Hep3B human HCC cell line

Overexpression or knockdown of KDM5B expression in the human HCC cell line, Hep3B, and the transfection efficiency was confirmed by Western blotting (Figure 3A). The characteristics of different transfected cells were investigated. KDM5B overexpression enhanced colony formation and cell proliferation when compared with KDM5B knockdown Hep3B cells (Figure 3B, 3C). These data suggested that KDM5B might contribute to the progression of human HCC by enhancing tumor cell growth.

## Discussion

Post-translational demethylation of lysine residues on histone tails is an important chromatin modification that is mediated by specific subfamilies of lysine demethylases (KDMs) and has roles in many cellular processes [22]. Mutations in the *KDM5A* gene in humans are associated with chronic inflammatory diseases [23,24]. Reduction of the expression

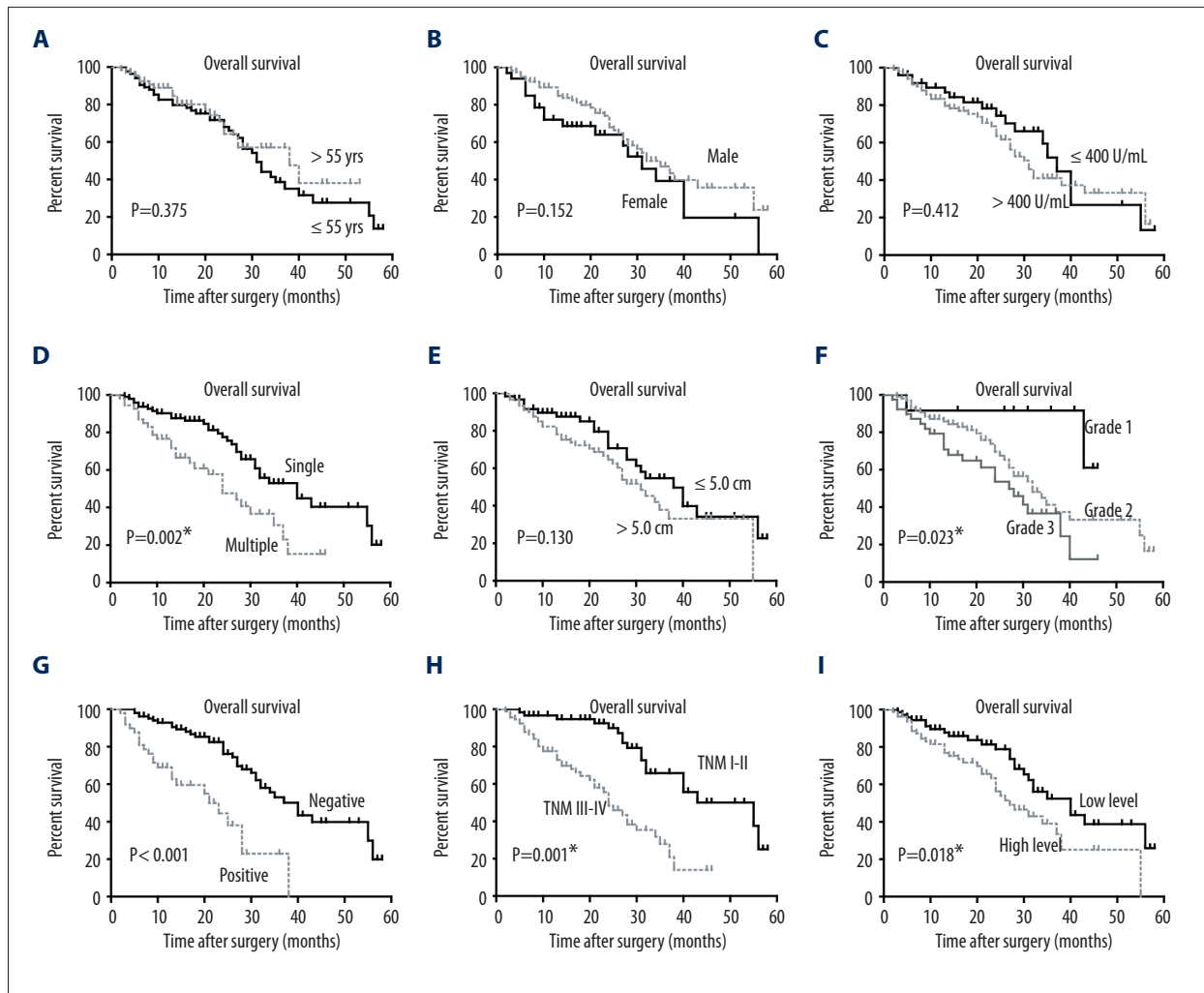
**Table 1.** Effects of KDM5B in HCC patients.

Variables	Cases			P value
	(n=152)	Low (n=72)	High (n=80)	
Age (years)				0.570
≤55	85	42	43	
>55	67	30	37	
Sex				0.520
Female	33	14	19	
Male	119	58	61	
Serum AFP				0.813
≤400 U/mL	50	23	27	
>400 U/mL	102	49	53	
Tumor number				0.058
Single	98	52	46	
Multiple	54	20	34	
Tumor size				<0.001*
≤5.0 cm	61	40	21	
>5.0 cm	91	32	59	
Pathological grade				0.730
Grade 1	12	7	5	
Grade 2	101	47	54	
Grade 3	39	18	21	
Portal vein invasion				0.264
Negative	103	52	51	
Positive	49	20	29	
TNM stage				0.012*
TNM I-II	60	26	24	
TNM III-IV	92	36	56	

\* Statistically significant by chi-square test.

of KDM5C has been shown to be associated with some neurodegenerative diseases [25]. Recent studies have shown that overexpression or mutations of KDMs are associated with the initiation and progression of several human cancers [26]. For example, overexpression of KDM4C is expressed in renal cell carcinoma [27]. Inactivation of mutations of the *KDM5D* gene has been shown to promote the progression of prostate cancer [28]. Emerging evidence from published studies has shown that members of the KDM5 family are involved in tumor development and progression, and may serve as novel cancer therapeutic targets [14,29].

KDM5B, which belongs to the KDM5 family, can function as a transcriptional suppressor by specifically removing methyl residues from lysine 4 of histone 3 (H3K4), and consequently suppresses gene transcription [30]. A recently published study has shown that KDM5B exhibited tumorigenic activity in a variety of human cancer types [31]. Several studies have shown that KDM5B has a role in both tumor initiation and progression, and that overexpression of KDM5B has been reported in several human malignancies, suggesting that KDM5B may be required for cancer cell development [32,33]. However, the potential role of KDM5B in the pathogenesis of HCC remains poorly understood.



**Figure 2.** Analysis of the overall survival of patients with patients with hepatocellular carcinoma (HCC). The overall survival (OS) curves plotted by Kaplan–Meier analysis and assessed by log-rank test, based on patient age (A), gender (B), serum alpha-fetoprotein (AFP) (C), tumor number (D), tumor size (E), pathological grade (F), portal vein invasion (G), TNM stage (H), and KDM5B protein level (I), respectively. \*  $P < 0.05$  by log-rank test.

In the present study, we demonstrated that KDM5B expression was significantly increased in liver tissues containing HCC tissues when compared with adjacent normal liver tissues by immunohistochemistry (IHC) and quantitative real-time polymerase chain reaction (qRT-PCR). Also, the associations between KDM5B expression and the clinical characteristics of patients with HCC who were included in this study were evaluated. The increased expression of KDM5B was significantly associated with larger tumor size and advanced TNM stage in patients with HCC. Also, using multivariate analysis, increased expression of KDM5B was identified as an independent prognostic factor for patients with HCC. Finally, *in vitro* studies using the human HCC cell line, Hep3B, was used to investigate the potential effect of KDM5B on HCC cells, which showed that overexpression of KDM5B in Hep3B cells promoted colony formation and tumor cell proliferation, which were reversed by

knockdown of KDM5B. These findings support that increased expression of KDM5B was associated with a more aggressive clinicopathological outcome for patients with HCC, possibly by promoting tumor cell growth.

Previously published studies support the findings of the present study. KDM5B expression has been shown to be significantly upregulated in glioma tissues and associated with poor clinical outcomes in glioma patients [34]. KDM5B has been shown to promote the progression of thyroid cancer through direct suppression of p21 [35]. In gastric cancer cells, KDM5B expression was shown to increase cell proliferation and invasion [36]. Also, KDM5B was previously identified as a potential oncoprotein, as the inhibition of KDM5B activity was shown to suppress tumorigenesis *in vivo* in tumor-bearing mouse models [37]. Therefore, the increased expression of the KDM5B protein is

**Table 2.** Overall survival of HCC patients.

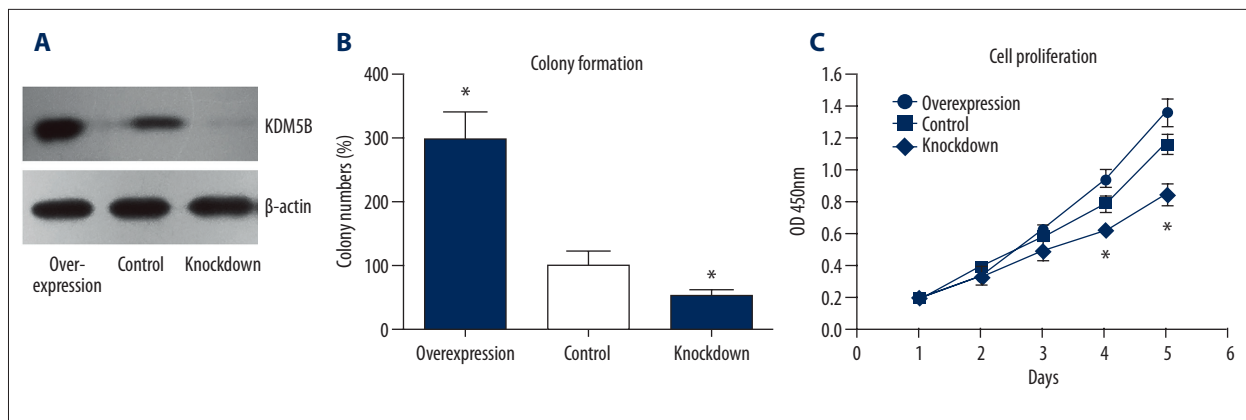
Variables	Patients (n=152)	Overall survival months		P value
		3-year OS	Mean ±S.D.	
Age (years)				0.375
≤55	85	38.6%	32.9±2.4	
>55	67	57.2%	35.0±2.9	
Sex				0.152
Female	33	39.3%	29.6±3.8	
Male	119	47.0%	35.8±2.3	
Serum AFP				0.412
≤400 U/mL	50	52.1%	35.5±3.2	
>400 U/mL	102	41.0%	33.4±2.3	
Tumor number				0.002*
Single	98	53.0%	38.4±2.4	
Multiple	54	30.6%	24.9±2.3	
Tumor size				0.130
≤5.0 cm	61	54.7%	37.2±2.9	
>5.0 cm	91	37.9%	32.3±2.5	
Pathological grade				0.023*
Grade 1	12	91.7%	41.7±3.3	
Grade 2	101	41.2%	34.9±2.5	
Grade 3	39	36.7%	25.9±2.5	
Portal vein invasion				<0.001*
Negative	103	53.0%	38.7±2.2	
Positive	49	22.8%	20.9±2.2	
TNM stage				<0.001*
TNM I-II	60	65.8%	43.5±2.6	
TNM III-IV	92	27.8%	24.8±1.8	
KDM5B level				0.018*
Low	72	52.4%	38.5±2.7	
High	80	39.1%	30.1±2.7	

\* Statistically significant by log-rank test.

**Table 3.** Multivariate analysis of HCC patients.

Variables	HR	95% CI	P value
Tumor number (multiple vs. single)	1.167	0.573-2.376	0.670
Pathological grade (grade 3 vs. 1/2)	1.330	0.770-2.300	0.307
Portal vein (positive vs. negative)	2.311	1.177-4.536	0.015*
TNM stage (III/IV vs. I-II)	1.369	1.015-3.753	0.044*
KDM5B level (high vs. low)	1.686	1.076-2.642	0.023*

\* Statistically significant by Cox regression model.



**Figure 3.** KDM5B promoted colony formation and proliferation of the hepatocellular carcinoma (HCC) cell line Hep3B. **(A)** Western blot results showed transfection efficiency of KDM5B plasmids and KDM5B-small interfering RNA (siRNA) in the hepatocellular carcinoma (HCC) cell line, Hep3B. **(B)** The results of colony formation assays performed in KDM5B overexpressing or siRNA knocked down cells. **(C)** Cell proliferation ability was tested in KDM5B overexpressing or in the siRNA knocked down cells.

significantly associated with the pathogenesis of malignant tumors. Further studies are needed to support these findings and to investigate the underlying molecular mechanisms for the roles of KDM5B in human malignancy, including HCC, to determine potential clinical applications.

## Conclusions

The findings of this study showed that KDM5B was highly expressed in tissues from patients with hepatocellular carcinoma

(HCC), and expression levels were significantly correlated with the poor clinicopathological features of tumors in patients with HCC. Also, using multivariate analysis, upregulation of KDM5B was identified as an independent negative prognostic factor for patients with HCC. These findings indicated that KDM5B requires further investigation as a potential novel prognostic biomarker in HCC.

## Conflict of interest

None.

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